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Autoreferát disertační práce

Proteomic analysis of soluble and transmembrane proteins in human lymphoma cells

Proteomická analýza rozpustných i membránových proteinů buněk lymfomu

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

Contents

Abstract

In the works presented here, we studied molecular changes associated with drug resistance in human mantle cell lymphoma (MCL) cells using proteomics. Our analyses allowed us to identify causal and/or secondary changes in protein expression associated with the development of resistance to the experimental drug TRAIL and the clinically used antimetabolites cytarabine and fludarabine. Resistance of MCL cells to the recombinant proapoptotic cytokine TRAIL was associated with downregulation of key enzymes of purine metabolism. This pathway potentially represents a molecular "weakness", which could be used as a therapeutic target for selective elimination of such resistant cells.

Resistance to the pyrimidine analog drug cytarabine was associated with cross-resistance to other antinucleosides. Proteomic and transcriptomic analyses showed pronounced downregulation of deoxycytidine kinase (dCK), which activates both purine and pyrimidine antinucleosides. This change explains the cross-resistance and is the causal mechanism of resistance to cytarabine. Our observations suggest that MCL patients, who do not respond to cytarabine-based therapy, should be treated with non-nucleoside drugs.

MCL cells resistant to purine-derived antinucleoside fludarabine were cross-resistant to all tested antinucleosides and also to ibrutinib, inhibitor of Bruton tyrosine kinase (BTK). Our proteomic analysis using a metabolic labeling approach (SILAC) showed marked downregulation of dCK and BTK among the differentially expressed proteins. Further, we detected upregulation of the anti-apoptotic protein Bcl-2, and demonstrated increased sensitivity of fludarabine-resistant MCL cells to the Bcl-2 inhibitor ABT199.

These "proof of concept" studies demonstrated the potential of proteomic analysis for personalized therapy of resistant malignancies. Proteomics, however, still has its limitations: the second section of this thesis deals with integral membrane proteins (IMPs). IMPs are underrepresented in conventional proteomic analyses, primarily due to their amphipathy, low digestibility with trypsin, and low expression levels. These

properties call for specific approaches. We introduced an improved and simplified method for IMP analysis that targets transmembrane segments of IMPs. We used this method to characterize the membrane proteome of a MCL cell line. We identified over 800 IMPs including several so-called “missing proteins”, that had not previously been observed on the protein level.

Key words: proteomics, mantle cell lymphoma, drug resistance, drug targets, integral membrane proteins, mass spektrometry

Abstrakt

V prezentovaných pracích jsme se s využitím proteomiky zabývali molekulárními změnami v buňkách lymfomu z buněk plášťové zóny (mantle cell lymphoma, MCL), souvisejícími s vznikem lékové rezistence. Identifikovali jsme tak kauzální a/nebo sekundární změny v proteinové expresi, spojené s rozvojem rezistence vůči experimentální molekule TRAIL a klinicky používaným antimetabolitům cytarabinu a fludarabinu. Odvozené buňky MCL rezistentní vůči proapoptotickému cytokinu TRAIL se vyznačovaly sníženou expresí klíčových enzymů metabolismu purinů. Tuto metabolickou dráhu tak lze považovat za „slabinu“, kterou by bylo možné využít coby terapeutický cíl k selektivní eliminaci takovýchto rezistentních buněk.

Rezistence vůči pyrimidinovému analogu cytarabinu se projevila křížovou rezistencí k dalším antinukleosidům. Proteomická a transkriptomická analýza ukázaly výrazně sníženou expresi deoxycytidin kinázy (dCK), jež je nutná k aktivaci purinových i pyrimidinových antinukleosidů. Tato změna vysvětluje křížovou rezistenci a je kauzálním mechanizmem rezistence vůči cytarabinu. Naše výsledky naznačují, že pacienti s MCL, u nichž selhala léčba založená na cytarabinu, by neměli být léčeni antinukleosidovými léčivy.

Buňky MCL rezistentní vůči purinovému antinukleosidu fludarabinu vykazovaly křížovou rezistenci vůči všem testovaným antinukleosidům a také ibrutinibu, inhibitoru Brutonovy tyrozinkinázy (BTK). Naše proteomická analýza provedená pomocí metabolického značení (SILAC) ukázala mimo jiné výrazně sníženou expresi dCK a BTK. Rovněž jsme detekovali zvýšenou expresi anti-apoptotického proteinu Bcl-2, a doložili zvýšenou citlivost těchto rezistentních buněk MCL k inhibitoru Bcl-2, ABT199. Tyto práce dokládají, že proteomika má potenciál pro určování vhodné terapie rezistentních nádorových onemocnění.

Proteomika má však stale určitá omezení, z nichž zásadní je nekompatibilita s integrálními membránovými proteiny (IMP). Tomuto tématu se věnuji v druhé části dizertační práce. IMP jsou v běžných proteomických analýzách neúměrně málo zastoupeny, především vlivem jejich amfipatie, nízké štěpitelnosti trypsinem a nízkých hladin exprese. Kvůli těmto vlastnostem jsou k analýze IMP potřeba

specifické postupy. V naší metodologické práci jsme představili vylepšenou a zjednodušenou metodu pro analýzu IMP, cílící na transmemránové úseky IMP. Touto metodou jsme charakterizovali membránový proteom buněčné linie MCL. Identifikovali jsme více než 800 IMP včetně několika tzv. „missing proteins“, které dosud nebyly detekovány na proteinové úrovni.

Klíčová slova: proteomika, lymfom z buněk plášťové zóny, léková rezistence, cíle léčiv, integrální membránové proteiny, hmotnostní spektrometrie.

1 Introduction

1.1 Proteomics

The word “proteome” denotes the entire set of proteins in a given cell type, tissue, or organism at the given time, including all “variants” resulting from alternative splicing and posttranslational modifications. Expression proteomics studies quantitative changes in proteomes under different physiological or pathological conditions, allowing identification of molecular mechanisms of various biological processes, including human diseases.

The “classical” proteomic approach uses two-dimensional electrophoresis (2-DE) of intact proteins and mass spectrometry (MS) for their identification. Complex protein mixtures are separated by 2-DE, which combines isoelectric focusing and SDS-polyacrylamide gel electrophoresis. Protein spots on the polyacrylamide gels are stained and subjected to densitometric image analysis. The spots with differential density are subjected to protein identification: proteins in cut-out spots are in-gel digested, resulting peptides are extracted and identified by MS. The determined masses of peptides or their fragments are compared with theoretical masses of tryptic peptides or their fragments of all protein/gene sequences present in a database to identify the protein (for review see Rabilloud *et al.*, 2010).

More advanced “shotgun” proteomic approach, is based on digestion of complex protein samples with a sequence-specific protease prior their separation. The resulting complex mixture of peptides is most often separated by 1-D or 2-D liquid chromatography (LC). On-line connection of LC with MS capable of tandem mass analysis (MS/MS) allows the identification of the peptides and proteins. Quantification in “shotgun” proteomic analyses is usually achieved using isotopic labeling.

This dissertation thesis presents four studies, focusing on two separate but related issues. For this reason, the thesis is divided into two sections. The first section presents three studies on drug resistance in mantle cell lymphoma that we performed with the use of quantitative proteomics. The second section deals specifically with

integral membrane proteins. Because of their physical and chemical properties and low expression levels, integral membrane proteins represent technological challenge for proteomics and require specific analytical strategies.

1.2 Mantle cell lymphoma and drug resistance

In the first three published works presented in the thesis, we studied drug resistance in mantle cell lymphoma (MCL) cells with the use of quantitative proteomics. Drug resistance currently presents one of the most crucial obstacles to effective treatment of cancer. Similarly to most malignancies, MCL initially responds to chemotherapy, development of drug resistance is, however, the main cause of therapeutic failure. Several mechanisms for drug resistance exist, some are more universal, as drug detoxification by chemical modification and active drug efflux by multidrug resistance transporters (Fodale *et al.*, 2011), and antiapoptotic changes, as deregulation of DNA damage response proteins (Wang *et al.*, 2012) or apoptosis regulatory proteins (Igney & Krammer, 2002). Moreover, activation of some proliferative signaling pathways and supression of proliferation inhibitors may also participate in preventing cancer (Rebucci & Michiels, 2013). Depending on the chemotherapeutic agent, other mechanisms of drug resistance mechanisms may include downregulation of receptors and transporters specific for the drug, their activating enzymes, upregulation of enzymes capable of specific inactivation of the drug, or mutation or deregulation of the drug target proteins.

MCL is a non-Hodgkin mature B-cell lymphoma, characterized by chromosomal translocation, which results in constitutive overexpression of cyclin D. Its annual incidence is relatively low (0.5/100 000 in the USA and Europe) and the median age at diagnosis is 68 years. (Cheah *et al.*, 2016). Typical treatment comprises of combination therapy often including cytrabine. The response is typically short-term and relapse of the disease is common. Relapsed (and refractory) MCL is an untreatable malignancy (Dreyling *et al.*, 2013), median survival being 5-7 years (Herrmann *et al.*, 2009). No standard therapy for patients with relapsed/refractory MCL exists. Elucidation of the molecular mechanisms of resistance is essential for efficient and knowledge-based therapy. Proteomic analysis of therapy-resistant tumor

cells may elucidate the mechanisms of drug resistance and provide additional detailed information on other molecular processes associated with the drug resistence or with secondary adaptive changes. Such information is a prerequisite for selection of novel drug targets.

2 Section I: Proteomic analyses of drug-resistant MCL cells

2.1 Identification of potential therapeutic molecular targets in TRAIL-resistant MCL cells

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a proapoptotic cytokine, expressed by NK cells and cytotoxic T-lyphocytes, which, upon binding to its death receptor, induces the extrinsic apoptotic pathway. Its recombinant soluble form posesses cytostatic and cytotoxic activity in malignant transformed cells, while non-transformed cells are resistant to TRAIL (Ashkenazi *et al.*, 1999). Since TRAIL death receptors are highly expressed in various malignancies including MCL, recombinant TRAIL is an attractive therapeutic molecule for cancer treatment (Dimberg *et al.*, 2013). However, as with most other anti-cancer drugs, development of resistance to TRAIL has been observed.

The most common mechanism of resistance to TRAIL is loss of expression or mutation of the death receptors, or inhibition of signaling in the intrinsic apoptotic pathway (Cheng *et al.*, 2006, Dimberg *et al.*, 2013). In this study, we aimed to identify the secondary changes in protein expression associated with acquired resistance to TRAIL in MCL cells. These specific changes can represent potential therapeutic targets or “weaknesses”.

2.1.1 Derivation of TRAIL-resistant cell line

HBL-2 cell line, an established model of MCL, is sensitive to TRAIL. By prolonged exposure to increasing doses of TRAIL, we developed a TRAIL resistant subclone designated HBL-2/R, which proliferated in up to 1000 ng/ml TRAIL in medium. Flow cytometry analysis of HBL-2/R showed, that TRAIL death receptors were

markedly downregulated in HBL-2/R compared to HBL-2. Therefore, downregulation of the death receptors was the likely mechanism of resistance (Cheng *et al.*, 2006).

2.1.2 Proteomic analysis of the HBL-2 and HBL-2/R cell lines

The derived TRAIL-resistant HBL-2/R cells along with the original HBL-2 cells were subjected to 2-DE. Over 800 protein spots were reproducibly detected on coomassie-stained gels. Significantly quantitatively changed spots (11 upregulated and 10 downregulated in HBL-2/R cells, see Figure 1 and Table 1) were subjected to identification using MALDI-MS.

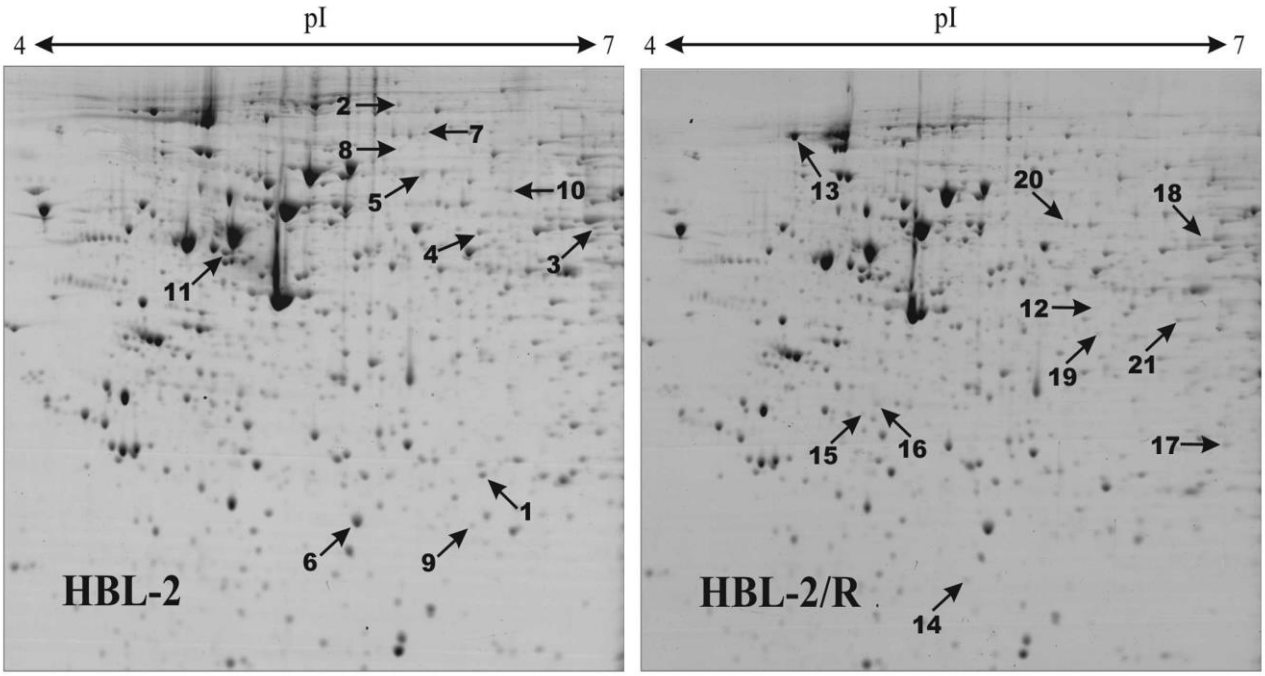

Figure 1. *Two-dimensional electrophoresis of HBL-2 and HBL-2/R cells stained with Coomassie Brilliant Blue. Differentially expressed proteins are indicated by numbered arrows (spots 1–11 indicate downregulated proteins and spots 12–21 indicate upregulated proteins in HBL-2/R cells).*

Among the differentially expressed proteins were three key enzymes of purine metabolism: adenine phosphoribosyltransferase (APRT, downregulatred 2.2-fold), Inosine-5′-monophosphate dehydrogenase 2 (IMPDH2, downregulated 1.6-fold) and mpurine nucleoside phosphorylase (PNP, downregulated 1.6-fold).

Table 1. *List of differentially expressed in proteins HBL-2/R cells (minimal 1.5-fold change and statistical significance p<0.05).*

Spot no.	Uniprot Acession	Protein name	Fold change	Mascot score	Sequence coverage (%)	Mol. weight
Proteins upregulated in HBL-2/R cells						
1	P04792	Heat shock protein β-1	3.9	84	51	22826
2	P42704	Leucine-rich PPR motif-containing protein	2.6	100	23	159003
3	O75351	Vacuolar protein sorting-associated protein 4B	2.6	171	32	49443
4	P23381	Tryptophanyl-tRNA synthetase	2.4	240	54	53474
5	P20591	Interferon-induced GTP-binding protein Mx1	2.2	176	42	75872
6	P09211	Glutathione S-transferase P	1.9	110	56	23569
7	P06396	Gelsolin	1.9	115	22	86043
8	P13010	X-ray repair cross-complementing protein 5	1.7	262	46	83222
9	Q9HAV7	GrpE protein homolog 1	1.6	99	44	24492
10	O43776	Asparaginyl-tRNA synthetase	1.5	250	41	63758
11	Q15084	Protein disulfide-isomerase A6	1.5	76	29	48490
Proteins downregulated in HBL-2/R cells						
12	P08559	Pyruvate dehydrogenase E1 component subunit α	3.2	111	32	43952
13	P19338	Nucleolin	2.4	146	29	76625
14	P07741	Adenine phosphoribosyltransferase	2.2	227	79	19766
15	O75792	Ribonuclease H2 subunit A	1.7	348	72	33716
16	Q07955	Serine/arginine-rich splicing factor 1	1.7	82	35	27842
17	P00491	Purine nucleoside phosphorylase	1.6	182	68	32325
18	P12268	Inosine-5'-monophosphate dehydrogenase 2	1.6	230	44	56226
19	P40121	Macrophage-capping protein	1.6	102	41	38760
20	P13674	Prolyl 4-hydroxylase subunit α-1	1.5	234	48	61296
21	Q15019	Septin-2	1.5	62	23	41689

2.1.3 Discussion

The downregulation of three key enzymes of purine metabolism identified by 2-DE-MS proteomic approach represents a non-causative secondary alteration, which may have a profound effect on nucleotide homeostasis in TRAIL-resistant lymphoma cells. Purine nucleotides, indispensable for DNA and RNA synthesis and as enzyme cofactors, are obtained by cells either by *de novo* synthesis, or by the so-called salvage pathway, a part of which are the three downregulated enzymes (see Figure 2). In leukemic and lymphoma cells, the salvage pathway is considered the major source of nucleotides (Scavennec *et al.*, 1982, Natsumeda *et al.*, 1984).

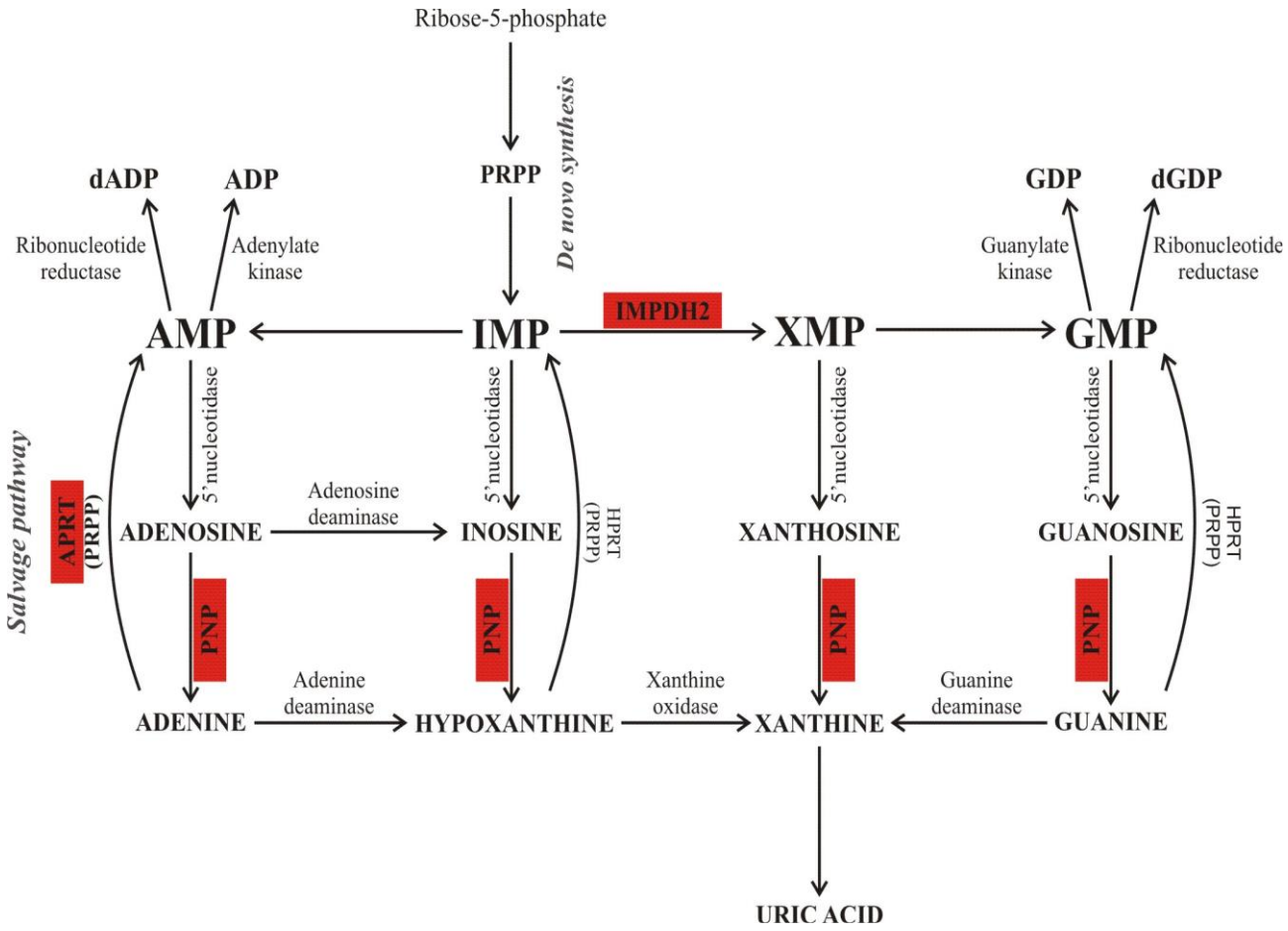

Figure 3.5. *Simplified scheme of purine metabolism, showing the position of APRT, IMPDH2 and PNP in purine nucleotide biosynthesis. The de novo synthesis of purine nucleotides begins with the phosphorylation of ribose-5-phosphate to form PRPP. In a number of reactions, PRPP forms the first fully formed nucleotide, IMP, which is converted by IMPDH2 to GMP. PNP catalyzes the reversible cleavage of purine nucleosides, releasing purine nucleobases (adenine, hypoxanthine, xanthine and guanine). In the salvage pathway the free nucleobases can be reconverted to nucleoside-5'-monophosphates in a reaction with activated sugar (PRPP) catalyzed by APRT.*

Changes in enzymatic activities and concentrations of the key enzymes of purine metabolism can lead to imbalance in purine nucleotide homeostasis in MCL cells. Accumulation or decrease of (deoxy)ribonucleotides or their intermediates can lead to inhibition of cell proliferation and induction of apoptosis (Galmarini *et al*., 2008, Hedstrom, 2009, Bollée *et al*., 2012). The impairment of purine metabolism in MCL cells therefore represents a selective disadvantage, or "weakness" of the resistant cells. Such "weakness" may be therapeutically exploited using inhibitors of purine metabolism. Several inhibitors of the enzymes participating in purine metabolism are already available for clinical use, e.g. methotrexate (inhibior of dihydrofolate

reductase), ribavirin and mycophenolic acid (inhibitors of IMPDH2) or forodesine (inhibitor of PNP).

In this study, we identified altered expression of several proteins including 3 enzymes of the purine metabolism pathway in a TRAIL-resistant MCL cell line. These molecular changes in the drug resistant cells (although not directly responsible for the resistance) represent a "weakness" that might be potentially used as a therapeutic target for the selective elimination of such resistant cells.

The results of this work were published in:

Resistance to TRAIL in mantle cell lymphoma cells is associated with the decreased expression of purine metabolism enzymes. Pospisilova J, Vit O, Lorkova L, Klanova M, Zivny J, Klener P, Petrak J. *International Journal of Molecular Medicine* 2013; 31(5):1273 (IF 2013: 1.880).

2.2 Elucidation of the mechanism of resistance to cytarabine in MCL cells

Cytarabine is a nucleoside antimetabolite used primarily in the treatment of leukemias and lymphomas, including MCL (Delarue *et al*., 2013). It is transported into the cells by equilibrative transporters ENT1 and ENT2 and also by passive diffusion. Phosphorylation of cytarabine by deoxycytidine kinase (dCK) to cytarabine monophosphate (ara-CMP) ensures that it is retained in the cell and allows its further phosphorylation to cytarabine triphosphate (ara-CTP). Incorporation of ara-CTP into DNA causes chain termination, thereby blocking DNA synthesis and inducing apoptosis. The development of resistance to cytarabine is common in MCL patients and leads to relapsed/refractory stage of the disease with poor prognosis. Currently there is no second line standard of care for relapsed/refractory MCL (Ferrero & Dreyling, 2013).

We derived several cytarabine-resistant MCL cell lines, tested their sensitivity to a battery of different anti-cancer drugs and performed a proteomic analysis of

the drug resistant cells. The goal of this work was to elucidate the causative mechanism of resistance cytarabine in MCL cells.

2.2.1 Derivation of cytarabine-resistant cell line and assesment of sensitivity to other drugs

Five established cell lines derived from MCL (GRANTA-519, HBL-2, JEKO-1, MINO and REC-1), all sensitive to cytarabine, were exposed to gradually increasing doses of cytarabine, up to 50 μM for several weeks. The resulting resistant subclones (GRANTA-519/R, HBL-2/R, JEKO-1/R, MINO/R and REC-1/R) tolerated up to 1,000-fold higher concentrations of cytarabine in media compared to the original sensitive cell lines.

We tested the sensitivity of the cytarabine resistant cells to a battery of different anti-cancer drugs both *in vitro* and *in vivo* using a panel of clinically used drugs: the alkylating agents bendamustine, cisplatin and doxorubicin, the nucleoside analogs cladribine, fludarabine and gemcitabine, and the inhibitory agents bortezomib (proteasome ihibitor), ibrutinib (Bruton tyrosine kinase, BTK) and temsirolimus (mammalian target of rapamycin, mTOR). *In vivo* toxicity tests were performed on immunodeficient mice (a model devoid of B and T cells, NK cells and complement, deficient of macrophages and dendritic cells) xenotransplanted with the MCL cell line JEKO-1 and the JEKO-1/R subclone.

Both *in vitro* and *in vivo* tests showed cross-resistance of the cytarabine resistant cells to both pyrimidine and purine derived nucleoside analogs, while sensitivity toward other drugs was retained. These results suggest a nucleoside-specific mechanism of the cytarabine resistance.

2.2.2 Proteomic and transcriptomic analysis of the resistant MCL cell lines

We subjected the newly established cytarabine resistant MINO/R cells to 2-D electrophoresis along with the original sensitive MINO cells. The gels were stained with Coomassie Brilliant Blue and following image analysis, spots with significant changes in density were in-gel digestied with trypsin and the extracted peptides were used to identify the differentially expressed proteins with MALDI-MS. In parallel

with the proteomic analysis, transcriptome profiling using Illumina BeadChips was performed for each of the five MCL cell lines and their cytarabine-resistant subclones.

Only one gene, *DCK* (coding dCK) was consistently differentially downregulated in all of the cell lines in the transcriptome analysis. In the proteomic analysis, one of the most markedly differentially expressed proteins was also dCK, downregulated in the MINO/R subclone (see Figure 3 and Table 2). The downregulation of dCK on protein level in all five cell lines and their resistant subclones was confirmed using Western blots (see Figure 4). Moreover, the lowered expression of dCK was also observed using Western blots in primary cells from patients that relapse after failure of high-dose cytarabine based treatment, or refractory to cytarabine treatment.

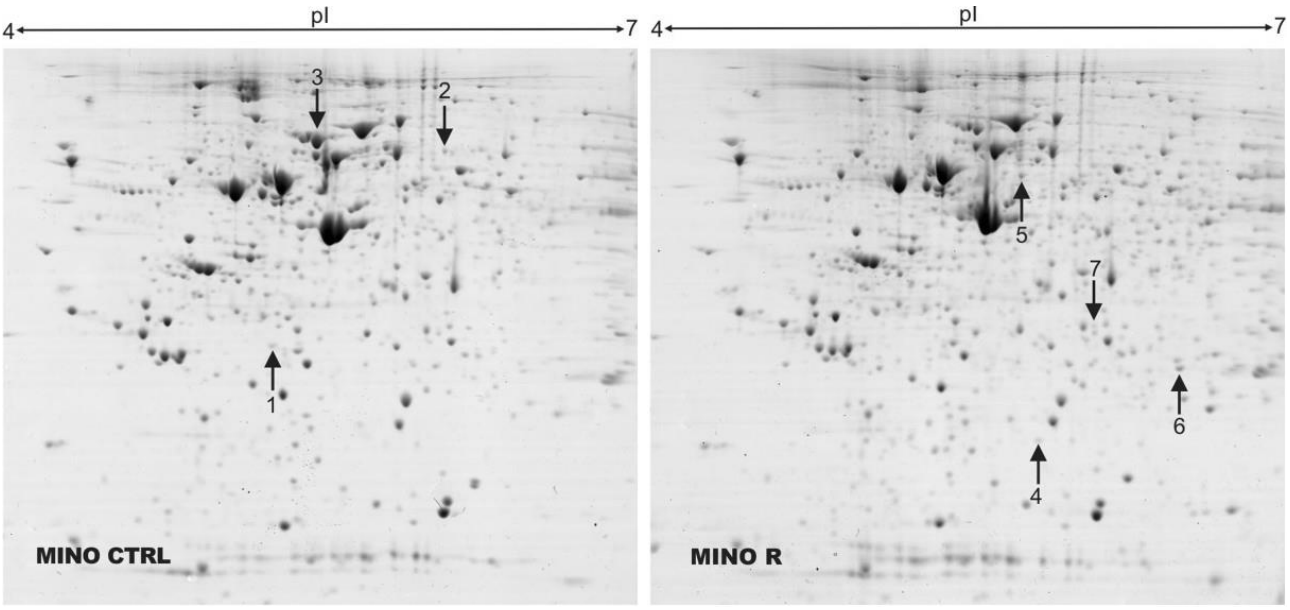

Figure 3. *Two-dimensional electrophoresis of MINO and MINO/R cells performed on 24-cm gel strips, pH 4.0–7.0 and 10% SDS-PAGE stained with Coomassie Brilliant Blue. Differentially expressed proteins are indicated by numbered arrows (spots 1–3 indicate downregulated proteins and spots 4–7 indicate upregulated proteins in HBL-2/R cells).*

dCK catalyses the first phosphorylation of nucleosides and nucleoside analogs, leading to their activation from a pro-drug to active drug form. This is a rate limiting step in the activation of cytarabine. dCK possesses low substrate specificity and phosphorylates both pyrimidine and purine nucleosides, as well as nucleoside analogs

(Arnér & Eriksson, 1995). Downregulation of dCK has been shown to be a causal mechanism of resistance to cytosine analog decitabine (Qin *et al*., 2009).

Table 2. *List of differentially expressed in proteins MINO/R cells (minimal 1.5-fold change and statistical significance p<0.05).*

Spot no.	Uniprot Acession	Protein name	Fold change	Mascot score	Sequence coverage (%)	Mr
Proteins downregulated in MINO/R cells						
1	P27707	Deoxycytidine kinase	4.6	44*	16	30841
2	Q99829	Copine-1	4.3	102	17	59649
3	P13796	Plastin-2	2	453	65	70814
Proteins upregulated in MINO/R cells						
4	P07741	Adenine phosphoribosyltransferase	5	70	40	19766
5	P68363	Tubulin α-1B chain	5	169	32	50804
6	P04792	Heat shock protein β-1	2.3	73	32	22826
7	P31937	3-Hydroxyisobutyrate dehydrogenase	2.1	43*	8	35712

* Identity of proteins with low Mascot score was verified by MS/MS:

Spot no.	Uniprot Acession	Protein name	Peptide sequences	Mascot score
1	P27707	Deoxycytidine kinase	LKDAEKPVLFFER QLCEDWEVVPEPVAR	41 46
2	P31937	3-Hydroxyisobutyrate Dehydrogenase	DFSSVFQFLREEETF SPILLGSLAHQIYR	49 28

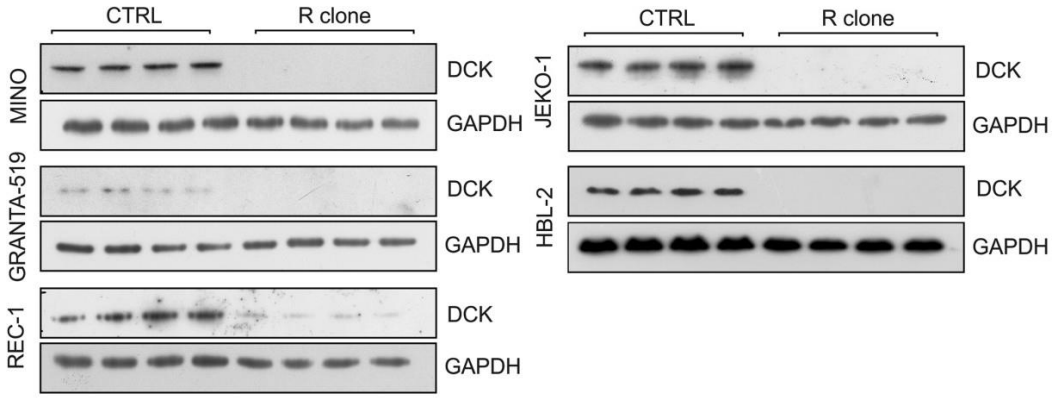

Figure 4. *Western blot verification of marked downregulation of dCK in all R-subclones. GAPDH was used as the loading control.*

Our results indicate, that patients with cytarabine-resistant MCL should not be treated with pyrimidine nor purine analogs, because the markedly decreased, if not totally

silenced, expression of dCK is most likely the molecular mechanism of resistance to cytarabine being also responsible for the cross-resistance to other antinucleoside drugs, both purine and pyrimidine derived. Instead, other types of anti-cancer drugs should be used.

The results of this work were published in:

Downregulation of deoxycytidine kinase in cytarabine-resistant mantle cell lymphoma cells confers cross-resistance to nucleoside analogs gemcitabine, fludarabine and cladribine, but not to other classes of anti-lymphoma agents. Klanova M, Lorkova L, Vit O, Maswabi B, Molinsky J, Pospisilova J, Vocková P, Mavis C, Lateckova L, Kulvait V, Vejmelkova D, Jaksa R, Hernandez F, Trneny M, Vokurka M, Petrak J, Klener P Jr. *Molecular Cancer.* 2014;13:159 (IF 2014: 4.257).

2.3 Functional and proteomic analysis of fludarabine resistant MCL cells

Fludarabine is a purine-derived nucleoside antimetabolite, which is being widely used for the salvage therapy of relapsed/refractory MCL (Forstpointner *et al.*, 2004). Fludarbine is transported into the cells by equilibrative nucleoside transporters, mainly ENT1 (Molina-Arcas *et al.*, 2003). Metabolic activation of fludarabine is carried out by phosphorylation by dCK (Danhauser *et al.*, 1986). This is the rate-limiting step for the formation of fludarabine triphosphate (F-ara-ATP). F-ara-ATP then directly inhibits DNA polymerases and is incorporated into the DNA strand. This results in chain termination, replication fork stalling, DNA breaks, and subsequent activation of DNA damage response, ultimately leading to apoptosis (Gandhi & Plunkett, 2002).

The goal of this work was to 1) identify the mechanism of fludarabine resistance, 2) to obtain as much as possible information about molecular changes in the fludarabine resistant cells and based on the information to propose novel strategies for the elimination of fludarabine-resistant MCL cells.

2.3.1 Derivation of fludarabine-resistant cell line and assessment of sensitivity to other drugs

MCL derived MINO cells were grown in the presence of increasing doses of fludarabine for prolonged period to derive fludarabine resistant subclone. Resulting MINO/FR subcloneproliferated in up to 100 μM fludarabine. We performed cytotoxicity tests using a panel of clinically used drugs in order to assess the sensitivity to several drug classes. In addition to fludrabine, the MINO/FR subclone displayed cross-resistance to all tested nucleoside analogs (cladribine, cytarabine and gemcitabine), and also to ibrutinib - an inhibitor of bruton tyrosine kinase (BTK).

2.3.2 Quantitative proteomic analysis of fludarabine sensitive and resistant cell lines

We performed a large-scale quantitative proteomic analysis of MINO and MINO/FR cells using stable isotope labeling in cell culture (SILAC, Ong *et al.*, 2002). The cells were supplemented with ^{13}C and ^{15}N-labeled arginine and lysine (heavy medium), or standard unlabeled amino acids (light medium). To provide sufficient robustness of the analysis, we set up “forward” and “reverse” experiments, where light and heavy media were interchanged. Cell lysates were subjected to filter-aided sample preparation (FASP, Wiśniewski *et al.*, 2009$^{\text{Nat Methods}}$) and resulting peptide samples were analyzed by LC-MS/MS. We identified 1,942 and 1,700 proteins in forward and reverse experiments, respectively. The expression ratios in forward and reverse experiments were highly correlated (see Figure 5), showing the high reproducibility of the used quantitative approach. Over 150 proteins were found to be downregulated and 160 upregulated with a fold change of at least 1.5

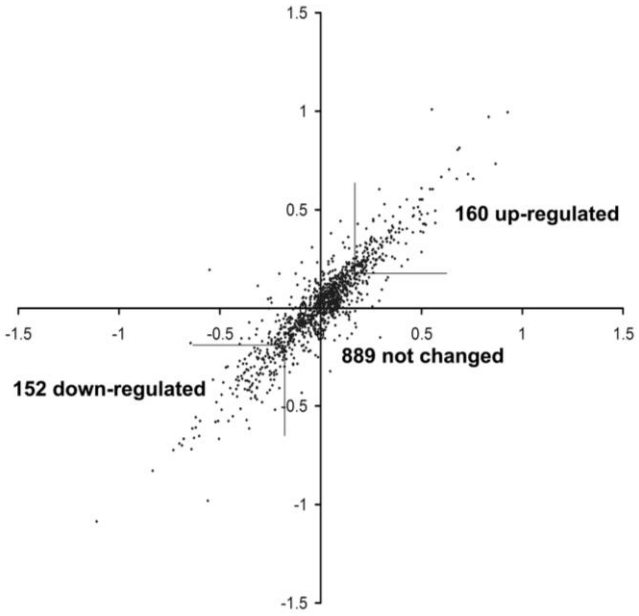

Figure 5. *Correlation of protein expression ratios in forward and reverse SILAC experiments. Log values of heavy/light protein ratios from the forward experiment are plotted against log values of light/heavy protein ratios from the reverse labeling experiment.*

Several-fold downregulation of dCK was one of the most prominent changes detected in our analysis. dCK phosphorylates and thus activates both pyrimidine and purine nucleosides and nucleoside analogs including fludarabine. The downregulation of dCK has previously been shown to be the mechanism of fludarabine resistance in leukemic cells (Månsson *et al*., 2003) and the mechanism of cytarabine resistance in MCL in our previous work. The massive downregulation detected in our analysis is therefore most likely the critical change responsible for fludarabine resistance in our MCL model and explains the observed cross-resistance to other antinucleosides. In addition to dCK, we detected upregulation of further enzymes of nucleotide *de novo* synthesis and interconversion, enzymes of serine biosynthesis, which can contribute to nucleotide *de novo* biosynthesis. The upregulation of numerous enzymes that play role in nucleoside metabolism may reflect the increased demand for deoxyribonucleotides (dNTP) after the loss of dCK expression.

Changes in protein expression that may lead to avoiding apoptosis included upregulation of numerous proteins that contribute to DNA repair, upregulation of negative regulator of apoptosis Bcl-2 and downregulation of its antagonist Bax. We tested the toxicity of ABT-199, the inhibitor of Bcl-2, to evaluate its potential for

the therapy of antinucleoside-resistant MCL. In MINO/FR cells, we observed markedly increased sensitivity of to ABT-199 compared to original MINO cell line.

Moreover, we also detected changes that could result in the loss of sensitivity towards other drugs. We observed downregulation of B-lymphocyte antigen CD20, which may limit the efficacy of chimeric anti-CD20 monoclonal antibody rituximab used in lymphoma therapy. Furthermore, we detected the strong downregulation of Bruton tyrosine kinase (BTK), which explains the loss of sensitivity of the fludarabine resistant cells to BTK inhibitor ibrutinib. This change, along with further downregulated proteins, suggests deregulation of B-cell receptor (BCR) signaling in MINO/FR cells.

2.3.3 Discussion

This work provides the evidence, that the downregulation of dCK is the likely mechanism of resistance of MCL cells to fludarabine and other antinucleoside drugs. The downregulation of BTK (associated with the decreased sensitivity of fludarabine-resistant cells to ibrutinib), the upregulation of Bcl-2 (responsible for an increased sensitivity to ABT-199) and the downregulation of CD20 (that may cause loss of sensitivity to rituximab) are further valuable information, which could be used to predict optimal treatment strategies in patients who fail fludarabine-based regimen.

Our analysis of mantle cell lymphoma model of acquired resistance to fludarabine thus provides a proof of concept that proteomics could be used in clinical settings for the prediction of optimal treatment strategies in near future. The characterization of small populations of cancer cells from individual patients may allow proteomics to contribute to the formulation of individualized therapies in the near future.

The results of this work were published in:

Detailed functional and proteomic characterization of fludarabine resistance in mantle cell lymphoma cells. Lorkova L, Scigelova M, Arrey TN, Vit O, Pospisilova J, Doktorova E, Klanova M, Alam M, Vockova P, Maswabi B, Klener P Jr., Petrak J. *PLoS One.* 2015;10(8):e0135314 (IF 2015: 3.057).

3 Section II: Proteomics of integral membrane proteins

Integral membrane proteins (IMPs) are encoded by roughly a quarter of human genes (Fagerberg *et al.*, 2010). IMPs execute important cellular processes, including transport, cell-cell interactions, signalling etc. This makes them attractive targets for therapeutic molecules, which is reflected in the fact that approximately half of the currently approved drugs in human medicine target IMPs (Yildirim *et al.*, 2007).

Standard proteomic approaches are unsuitable for α-helical IMPs for three major reasons: 1) α-helical IMPs are amphipathic and therefore insoluble in aqueous buffers, 2) lysine and arginine, cleaved by the most widely used protease trypsin, are relatively rare in the sequences of IMPs, 3) IMPs frequently exhibit low expression levels**.** For these reasons, IMPs are underrepresented in conventional proteomic analyses. **Specific approaches, that reflect the different physico-chemical properties of IMPs, are therefore needed to access the membrane proteome.**

We reviewed the available tools, which assist the proteomic analyses of membrane proteins, namely detergents, organic solvents, chaotropes, alternative cleavage strategies and the so called "divide and conquer" approach (targeting selectively only hydrophilic or hydrophobic segments of IMPs) in a **review paper included in the thesis, published in:**

Integral membrane proteins in proteomics: How to break open the black box? Vit O, Petrak J. *Journal of Proteomics*. 2016;153:8-20. (IF 2015: 3.867).

3.1 Development and application of a new method for proteomic analysis of integral membrane proteins based on their transmembrane segments

One of the successful strategies in the anlysis of IMP is the so called **"divide & conquer" approach**. This streategy circumvents the problem with amphipathy of IMPs by targeting either the hydrophilic, or hydrophobic segments of IMPs. The most successful "divide & conquer" strategies that target hydrophilic extra-membrane domains of IMPs are three strategies based on enrichment of glycoproteins

or glycopeptides. These are “cell surface capture”, which is based on biotin labeling of sugar moieties of cell surface membrane glycoproteins on intact cells (Wollscheid *et al.*, 2009). A reverse “divide & conquer” approach has been introduced by Blackler *et al.*, 2008: involving isolation of intact membranes, their washing in carbonate buffer and proteolytic digestion of accessible protein material allows isolation of highly enriched transmembrane segments of IMPs. We followed the path indicated by Wu & Blackler and modifed and improved their protocol.

The original protocol by Wu & Blackler abbreviated as hppK-CNBr (high pH, proteinase K, CNBr) employs non-specific proteinase K, which produces numerous overlapping peptides, leading to excessive sample complexity. A sequence specific protease such as trypsin would preclude this complication and would also enable a quantitative analysis of IMPs, for instance in connection with SILAC. In our work we a) modified the membrane isolation and IMP enrichment b) used trypsin instead of proteinase K c) optimized the conditions of peptide cleavage with CNBr and d) applied an alternative method of sample delipidation. In a reference to the original “hppK” method, we propose the acronym “hpTC” for our approach (high pH, Trypsin, CNBr). We applied the hpTC strategy to the analysis of IMPs of human lymphoma cells.

3.1.1 Optimization of the membrane isolation and digestion protocol

Instead of multi-step ultracentrifugation steps for membrane enrichment, we employed cell homogenization with a hypodermic needle, sedimentation of the unbroken cells and nuclei at 500×*g*, DNAse treatment to prevent the co-isolation of chromosomal DNA and subsequent sedimentation of the crude membrane fraction in a bench-top centrifuge at 18,000×*g*. To purify the membranes of human lymphoma cells, we applied carbonate washing at 4 °C, which allows opening of the membranes, and added trypsin to the resulting washed membrane pellet. After digestion, repeated carbonate washing and freeze-thaw cycles were done to remove digested non-membrane peptides. The effectiveness of this digestion procedure is visible on Figure 5C: the membrane vesicles that undergone the digestion are more sharply defined.

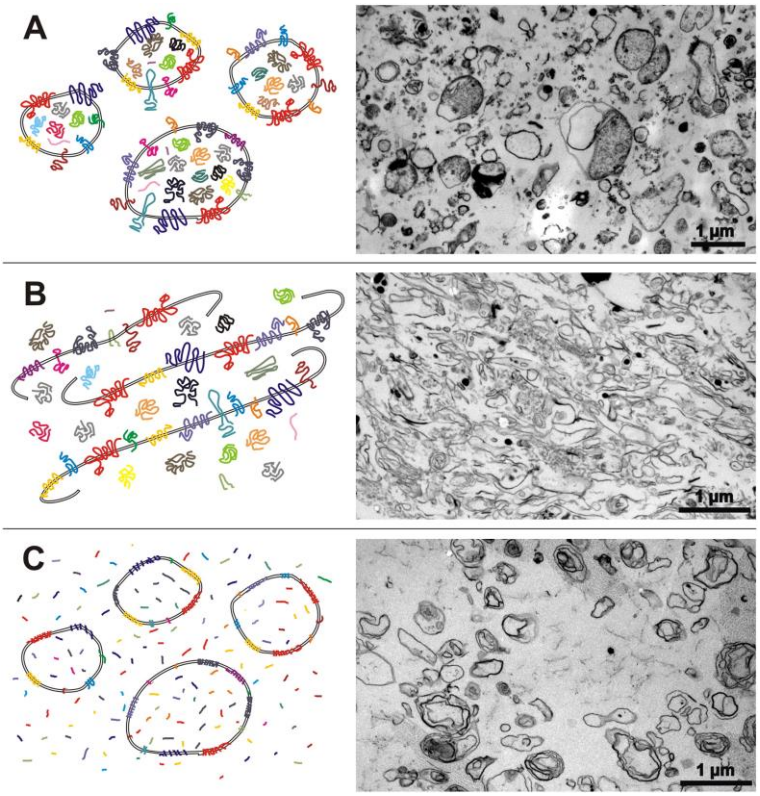

Figure 5. *Isolation (A), carbonate stripping (B) and proteolytic shaving (C) of membranes steps visualized by transmission electron microscopy (TEM). After the proteolytic digestion, the membrane vesicles are more sharply defined.*

We solubilized the transmembrane peptides in 70% trifluoroacetic acid instead of 90% FA to avoid formylation of the sample. After chemical cleavage with CNBr, the delipidation was done on reversed-phase column using dichloromethane washes, originally designed for removal of non-ionic detergents (Rey *et al.*, 2010), instead of lipid precipitation step, done in the original hppK-CNBr method, which can lead to loss of hydrophobic peptides.

3.1.2 Proteomic analysis and bioinformatic assessment of the results

LC-MS/MS analysis of the human lymphoma cell membrane sample generated by hpTC was performed using nano UHPLC with a 50 cm column and Hybrid Quadrupole-Orbitrap mass spectrometer Q Exactive plus (Thermo Fisher Scientific). In two runs, 1224 proteins were identified, 802 (65.5%) of the identifications contain at least one transmembrane domain according to TMHMM (Tied Mixture Hidden Markov Model, Krogh *et al.*, 2001) prediction. The identified IMPs contained between 1-16 transmembrane segments. The distribution of transmembrane segments

was in agreement with a genome-wide prediction of human proteome (Fagerberg *et al.*, 2010), suggesting that hpTC is not biased against more hydrophilic, nor the most hydrophobic IMPs with many TM segments (see Figure 6). Roughly half of the unique peptides belonging to IMPs overlapped with the predicted transmembrane segments. The importance of CNBr cleavge for the protocol is apparent from the high contribution of CNBr cleaved peptides to the identification of IMPs.

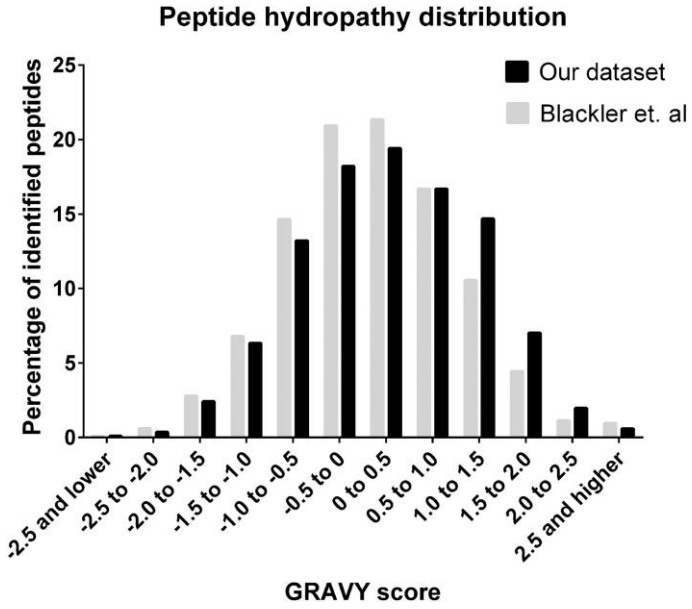

Figure 6. *Comparison of the distribution of peptide GRAVY score in our dataset with the results of the original method by Blackler et al. Distribution of peptides in our dataset is shifted toward higher GRAVY score (more hydrophobic peptides), presumably due to different method of sample delipidation.*

Among the 802 IMPs identified in the human lymphoma cells were numerous transporters, membrane enzymes, receptors and signal transduction proteins, proteins with immunity-related activities and other proteins. High abundant subunits of the inner mitochondrial electron transport chain complexes were among the IMPs with highest number of identified peptides. IMPs from all membraneous cellular compartments were identified. Besides known proteins, we identified 13 so called "missing proteins," gene products that have not been previously detected on protein level. Despite the presence of contaminating soluble proteins in our analysis results, two thirds of identified proteins were IMPs. This enrichment of IMPs is high, compared to conventional proteomic analyses of membrane proteins, usually ranging between 20-40% of IMPs, and demonstrates high efficacy of the hpTC method.

Implementation of trypsin instead of proteinase K decreases the sample complexity and also makes the method compatible with SILAC labeling. Nearly 83% of peptides contained SILAC-suitable amino acids arginine (R) or lysine (L). With addition of labeled leucine (L), which by itself also accounts for 83%, the “triple” labeling with R, K and L would provide semi-quantitative data on 97% of all identified peptides.

3.1.3 Discussion

We demonstrated that our modified hPTC strategy, combining trypsin digestion of intact membrane fraction with CNBr cleavage of trypsin protected transmembrane segments enables very high enrichment and analysis of hundreds of IMPs from all cellular compartments.

We modified several steps of the original method presented by Blackler & Wu. In particular, we excluded the use of ultracentrifugation, we used deoxyribonuclease for the removal of co-isolated chromosomal DNA and most importantly, we replaced the non-specific proteinase K with trypsin, which decrases sample complexity and opens a way toward combining this method with SILAC or label-free quantification. We also employed on-column sample delipidation with dichloromethane.

Our method is not limited to glycosylated IMPs of plasma membrane, as are glyco-capture methods. By focusing on membrane-embedded segments of IMPs, the method enables effective enrichment of IMPs. In combination with the dual trypsin-CNBr cleavage, this allows identification of otherwise problematic small, hydrophobic and low-abundant proteins. Taken together, this method is well suited for sensitive analysis of membrane proteome.

The results of this work were published in:

Large-scale identification of membrane proteins based onanalysis of trypsin-protected transmembrane segments. Vit O, Man P, Kadek A, Hausner J, Sklenar J, Harant K, Novak P, Scigelova M, Woffendin G, Petrak J. *Journal of Proteomics* 2016;149:15-22. (IF 2015: 3.867).

4 Conclusions

In the works presented in this thesis, we demonstrated that proteomics can provide detailed insights into quantitative changes in the proteomes of drug resistant cancer cells. Namely, we identified causal and secondary contributing or compensatory changes in MCL cells with acquired resistance to three different anti-cancer drugs. Detailed knowledge of the molecular landscape of drug-resistant cancer cells is a prerequisite for successful therapy. Based on the information obtained by proteomics, we were able to propose appropriate therapeutic strategies, or conversely, predict which drugs would be ineffective in the treatment of drug resistant cells. We believe that our works provide a proof of concept that a detailed proteomic analyses of small populations of cancer cells can be used in the clinical setting and direct individualized therapies in the near future.

Despite the enormous progress in proteomic technologies in the last decade, there are still shortcomings that need to be addressed. The inadequacy of standard proteomic workflows for integral membrane proteins is one of the most insistent. New paradigms and innovative approaches are needed. We followed an unexplored path toward the analysis of the membrane proteome, and presented a modified and improved method for the analysis of membrane proteins via their membrane-embedded peptides. This method, abbreviated as hpTC, allows the detection of the least-accessible, less-abundant and highly hydrophobic IMPs. We applied the method in the analysis of the membrane proteome of MCL cells. IMPs are an important part of the proteome and physiology of cells and the majority of current drugs target IMPs. Methods such as hpTC capable of accessing the membrane proteome are therefore essential to biomedical research. We believe that future applications of hpTC may provide new insights into biomedical problems including cancer drug resistance, identification of sensitive diagnostic markers and suitable drug targets.

5 List of publications

Publications, which are the basis of the thesis

Pospíšilová J, Vít O, Lorková L, Klánová M, Živný J, Klener P, Petrák J. Resistance to TRAIL in mantle cell lymphoma cells is associated with the decreased expression of purine metabolism enzymes. International Journal of Molecular Medicine 2013; 31(5):1273 (IF 2013: 1.880).

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